EFFECTS OF THREE CONCENTRATIONS OF METHOPRENE ON OVIPOSITION SITE SELECTION AND EGG HATCH RATE IN *AEDES TRISERIATUS* SAY

An Honors Thesis (HONRS 499)

by

Janel J. Stewart

Thesis Advisor
Dr. Robert R. Pinger

Robert R. Pinger

Ball State University

Muncie, Indiana

July 23, 1993

July 24, 1993

ACKNOWLEDGEMENTS

I would like to express my appreciation to Dr. Robert R. Pinger, who gave me his advise, his time, and his expertise, as well as the use of his facilities. His assistance was of utmost importance in the completion of this project. I would also like to thank Dr. Larry Henriksen for providing the statistical data necessary for the results of this procedure.

ABSTRACT

Laboratory experiments were conducted to determine the attractiveness of methoprene to ovipositing female *Aedes triseriatus*, and to determine whether eggs laid on methoprene impregnated substrate would hatch at the same rate as those laid on normal untreated substrate. Three concentrations of methoprene were used: 0.1 x the normal field application rate, the normal field application rate and 10 x the normal field application rate. Results failed to demonstrate any significant differences in the attractiveness of the sites. Results of the hatching also revealed no significant differences in the hatch rates of batches of eggs laid on any of the substrates.

INTRODUCTION

A laboratory experiment was conducted to test the effectiveness of methoprene as an oviposition attractant to gravid *Aedes triseriatus* females and to determine if methoprene would have an effect upon the hatch rate of those eggs deposited. If it could be shown that methoprene was especially attractive to the gravid females, or that it could reduce the hatch rate of eggs deposited on methoprene saturated substrate; these effects could be considered bonus effects to the larvacidal effects already reported for this material.

Eggs were collected by placing four oviposition sites, three containing different concentrations of methoprene and one control, into the cage. The total number of eggs deposited on each site was counted and recorded. The eggs were later hatched using deoxygenated, distilled water, and the number of larvae present were counted to obtain the hatch rate.

Null Hypothesis (1) Methoprene is not an oviposition attractant.

Null Hypothesis (2) Methoprene has no affect on the hatch rate of *Aedes triseriatus*

LITERATURE REVIEW

Aedes triseriatus Say

Aedes triseriatus Say is the primary vector for LaCrosse (LAC) encephalitis virus, one of the California Serogroup viruses.[4,5] *Ae. triseriatus* inhabits areas east of the Great Plains (See Figure 1), including the Canadian Provinces of Ontario, Quebec, and New Brunswick.[6] This mosquito is of particular importance in the north central United States where the most cases of LAC encephalitis have been reported (See Figure 2). This mosquito breeds in tree holes and in artificial containers that hold water and debris, including discarded automobile tires, tin cans, wooden barrels and watering troughs.[7,8] The eggs of *Ae. triseriatus* are oviposited onto the side of the treehole or container, just above the water line. These eggs will then hatch when they are covered with water and are at a favorable temperature.

Characteristics of the *Aedes triseriatus* Egg

The egg of *Ae. triseriatus* is dull black in color and has the shape of a football. The eggs are undefinable to the naked eye due to their minuscule size; the average length of an egg is approximately 680.8 μm and the average width is 201.6 μm.[9] Hundreds of eggs can fit in a small area. There are 816 eggs are pictured on a paper towel oviposition site in Figure 3.

The outer chorionic cells surrounding the egg are hexagonal in shape. These cells have a component which gives the *Aedes triseriatus* egg its most characteristic feature. There are one to three large tubercles pressed against the elevated wall of the chorionic reticulum. These tubercles are usually found on the same side of adjacent cells, causing rows to form on the egg surface.

LAC and other California Encephalitis Group Viruses

California encephalitis is a generic term referring to encephalitis resulting from any one of nine viruses in the California Serogroup (genus *Bunyavirus*, family *Bunyaviridae*). There are twelve California Serogroup viruses; the following nine cause human disease: California encephalitis (CE), LaCrosse (LAC), snowshoe hare (SSH), trivittatus (TVT), Tahyna (TAH), Lumbo (LUM), Inkoo (INK), Melao (MEL) and Guaroa (GRO).[10] Of these nine viruses, the first four are known to cause encephalitis in North America, but most cases have been attributed to the LAC virus.[11]

California encephalitis was first identified in 1943 when few arboviruses were known. The virus was isolated from mosquitoes by Hammon and Reeves, and described as a neurotropic virus that multiplied and circulated in the blood. It was classified as a member of arthropod-borne encephalitis viruses.[12] The first isolation from a human being occurred in 1960 in LaCrosse, Wisconsin, from the brain of four-year-old girl who had died of meningoencephalitis.[7]

In 1952, a screening in Kern County, California, found an 11% prevalence of

antibodies for California Encephalitis Virus (CEV). By 1963, when another screening was completed, this prevalence of antibodies had increased to 35%.[13] From 1964 to 1981, 36.2% of encephalitis cases in the United States were found to have resulted from California Serogroup viruses. This percentage was second only to the percentage due to St. Louis encephalitis virus. These encephalitis cases originated in twenty-one states, but 94.8% of these cases were from the following seven states: Illinois, Indiana, Iowa, Minnesota, New York, Ohio and Wisconsin.[10]

Pathways of infection of LAC virus in *Aedes triseriatus*

There are two important pathways of natural infection in *Ae. triseriatus*. The first is by ingesting a blood meal that contains the virus. In this respect, several mammals are indirectly involved in the cycle of the LAC virus transmission. High antibody prevalence (65-75%) has been found in the eastern gray squirrel, western fox squirrel and in chipmunks, while moderate antibody prevalence (15%) has been found in cottontail rabbits.[14] This pathway is called the vector-virus cycle and is illustrated in more detail in Figure 4. The second pathway of infection in *Ae. triseriatus* makes it unique from other mosquito-borne encephalitic viruses in that they are able to transmit the virus transovarially. This means that the female transmits the virus to her progeny through the egg. This mechanism allows the virus to survive throughout the winter and is therefore referred to as "overwintering".[5,15,16] This overwintering mechanism adds a mosquito-mosquito cycle to that of the vector-virus cycle and

permits the prevalence rate of infection to remain steady from year to year, instead of declining when there is no vector activity.

With the continuing threat of the mosquito-borne encephalitides, particularly LAC virus in the midwest, there has been considerable interest in mosquito control as well as other options; these options include public education of the problem, physical mosquito control, such as removing artificial containers and filling tree holes where the mosquitoes may breed, biological mosquito control using microorganisms and chemical control using pesticides. The most permanent control methods would be to eliminate breeding grounds for the mosquitoes entirely. Efforts in LaCrosse County, Wisconsin using these physical controls have had good, but slow, results.[17] However, in most places, chemical control methods are still the mainstay of vector control.

Altosid® (Methoprene)

Altosid® (methoprene), is a chemical larvicide that is thought to interfere with the normal mosquito growth cycle and prevent larvae from developing into adults. It is at this adult stage that the mosquito is capable of transmitting disease; therefore, the fewer mosquitoes emerging to adults results in fewer potential viral vectors.

Methoprene has been shown to be effective in controlling a variety of mosquito species. Several areas in Kenya were treated with sustained-release Altosid® (methoprene) pellets at different intervals prior to flooding in the area, and it was found that the methoprene effectively prevented emergence of *Aedes mcintoshi, Aedes*

dentatus, Aedes comminsii, and *Aedes circumluteolus.* Areas treated with methoprene five weeks prior to flooding showed 98% mortality, while areas treated one day after flooding showed 100% mortality. In contrast, the control (untreated) area had more than 99% of the pupae emerging to adults.[1]

Another study was engineered using the same type of methoprene in field plots in the laboratory. Two concentrations of methoprene were used: 2.2 kg/ha and 4.5 kg/ha. This study found that the lower concentration of methoprene effectively reduced emergence of *Ae. taeniarhynchus* for about 3 weeks, but after the 3 week period, there was a significant decrease in its effectiveness. The higher concentration of methoprene, however, still showed a 98% mortality rate even 34 weeks after the treatment.[2]

Beehler and Defoliart found that vegetable dye added to water acted as an oviposition attractant. The vegetable dye in the water increased the optical density and significantly increased the number of eggs deposited onto traps in the field containing vegetable dye.[3] It has been suggested that certain concentrations of methoprene might actually be attractive to gravid female mosquitoes, thus enhancing its effectiveness as a control method.

Objectives and Hypotheses

If the methoprene could be shown to be attractive to ovipositing *Ae. triseriatus* females, this would add to its effectiveness as a mosquito control agent. The objectives of this experiment are two-fold: 1.) to determine whether there is a significant difference between the attractiveness of methoprene-impregnated oviposition sites and distilled water-impregnated sites, and 2.) to test the ability of three different concentrations of methoprene, when applied to the oviposition substrate, to lower the hatch rate of *Ae. triseriatus* eggs.

Null Hypothesis (1) Methoprene is not an oviposition attractant.

Null Hypothesis (2) Methoprene has no affect on the hatch rate of *Aedes triseriatus*

MATERIALS AND METHODS

A colony of *Ae. triseriatus* (Walton strain) was established with eggs supplied by Dr. George B. Craig, Jr. of the University of Notre Dame. The colony was maintained in an environmental chamber (Percival®, model WE-95) at 25°C and 80% humidity, with a light:dark photoperiod of 14:10. Adults of the colony were provided access to a 10% sucrose solution, except for seven-hour period immediately prior to being offered a blood meal.

Hatching Eggs:

Deoxygenated water was prepared by autoclaving distilled water at 250°F for 15 minutes. The eggs deposited on paper toweling were hatched by inserting the toweling into 1 quart jars containing the deoxygenated water at room temperature. At the end of approximately eight hours, the unhatched eggs were removed and any hatched larvae were counted using a dissecting microscope and a hand-held counter.

Maintenance and Feeding of Mosquitoes:

The larvae were reared in pans with plexiglass covers in the environmental chamber. They were fed ground Tetramin® fish food. Pupae were removed from the pans and placed in fingerbowls inside 18" x 18" x 18" aluminum cages. Erlenmeyer flasks (250 ml) with cotton wicks were filled with a 10% sucrose solution and placed

in the center of each cage as an energy source for the emerging adult mosquitoes. These sucrose solutions were replaced every 3-4 days.

The flasks containing the sucrose were removed approximately 7 hours prior to the time when the mosquitoes were to be fed a blood meal. A guinea pig was used for the first two experimental runs and a rabbit was used for the remainder of the runs as a blood meal source. The animals were immobilized by injecting them intramuscularly with a combination of PromAce® (Acepromazine maleate) and Ketaset® (Ketamine HCl). They were shaved and laid on top of the cage containing the adult mosquitoes.

Experimental Procedure:

Approximately 150 male and 150 female pupae were placed in a fingerbowl, a 4.6 cm x 4.6 cm x 4.6 cm (18 in^3) cage, and allowed to emerge to adults. The adults were provided with a sucrose solution as described above. One week after the completion of adult eclosion, the sucrose solution was removed in preparation for the blood meal.

Approximately 72 hours following the blood meal, oviposition sites were inserted into the cages. The oviposition sites were rotated clockwise with each repetition to minimize any factors, such as side to side preference or lighting differences, that could have affected the oviposition site selection.

Oviposition sites

Four experimental oviposition sites were placed into the cages at equal distances around the sucrose solution. Oviposition sites consisted of brown paper toweling wrapped inside a 300 ml beaker containing 200 ml of water and 1 ml of a test solution. The solution in the oviposition sites contained three different concentrations of methoprene and one control. The sites were numbered one to four and contained the following: #1) control solution of ethanol, #2) methoprene at 1/10th the standard application rate, #3) methoprene at the standard application rate, and #4) methoprene at 10 x the standard application rate.

Testing Methoprene as an oviposition attractant

When oviposition was complete, the oviposition sites were removed and the eggs counted under a dissecting microscope. They were then stored in plastic Ziploc® bags in the environmental chamber. The purpose in the counting of the eggs was to determine whether ovipositing females preferred particular oviposition sites.

Eggs were incubated a minimum of one week before they were hatched. The eggs were hatched following the procedure outlined above. The larvae were then counted, once again using the dissecting microscope, to determine if the hatch rate of the eggs from different oviposition sites varied. The number of living and dead larvae were recorded to ascertain if there was a difference in the viability of those larvae from the four different oviposition sites. This procedure remained the same for all

fifteen repetitions with only a few exceptions that are mentioned in the discussion below.

RESULTS

oviposition preference

Table 1 summarizes the results of the experiments. A substantial number of eggs were observed in each of the three concentrations of methoprene and in the control. Statistical analysis of all egg counts using ANOVA failed to demonstrate a significant difference in the number of eggs deposited at any particular site in the cage or at any particular concentration of methoprene or the control. An F value of .175 was generated by ANOVA with an associated P value of .913, which is much higher than the critical P value of .05 that was needed to reject the null hypothesis.

hatch rate of eggs

The number of eggs laid, number hatched and hatch rate for each concentration of chemical and for the control are also shown in Table 1. There was no significant difference in hatch rate shown among the various treatments or the control. ANOVA generated an F value of 1.098 with an associated P value of .359, also much greater than the critical P value of .05 needed to reject the null hypothesis.

DISCUSSION

The results showed that there were no significant differences in the oviposition site selection or hatch rate of *Ae. triseriatus* when oviposition substrates were impregnated with methoprene. Similar results were reported by Beehler and Mulla, who tested the attractiveness of methoprene on the ovipositional behavior of *Aedes aegypti* and *Culex quinquefasciatus*. They also found no significant difference between the number of eggs laid in normal water and in water treated with methoprene.[18] The homogeneity of sites with regard to oviposition preference in the present study is shown in Figure 5. It perhaps is of some value to know that even at 10 x the standard field application rate, methoprene did not repel oviposition females.

The average hatch rates for each oviposition site differed only slightly (see Figure 6); there differences were not significant. Again, the results are consistent with those of Beehler and Mulla.[18]

Two weaknesses were found in our procedure during the first two trials that led to modifications for the remaining thirteen repetitions. The first problem had to do with egg storage. In the first two runs, eggs became desiccated by the time they were to be hatched, resulting in undue mortality. For this reason, eggs collected in subsequent runs were stored in plastic Ziploc® bags to retain moisture and a humid environment. The second problem was that the mosquitoes did not feed satisfactorily on the guinea pig. A rabbit was used as a source of a blood meal for the mosquitoes in the latter runs and seemed to work much better.

There were also several differences noted in the results of a few experimental runs. The first occurred in the run of 1/29. A batch of eggs received from Dr. George B. Craig, Jr. were hatched the same day as this experimental run and had pupae appearing after eight days. The first pupae from the experimental run, however, did not appear until thirteen days later. This pupae came from the control oviposition site and it was another five days (18 days total) before pupae appeared in the three sites containing methoprene. The larvae in this experimental run remained smaller than normal throughout their development.

A similar phenomena was observed in the run of 5/27, but only in the 1 x normal field application of methoprene. It took 22 days for the pupae to appear in this batch while the other sites had pupae which had even emerged to adults by day 16.

On a few runs, the larvae were reared in the quart jars that the eggs had been hatched in. The run of 2/26 was one of those. Six days after hatching, the water containing the larvae of the control batch was clear, but the water containing larvae from the three methoprene batches was very turbid (See Figure 7). All four batches had been fed approximately the same amount of tetramin® at the same times. The cause of this turbidity is unknown, but pans were once again used to rear the larvae in after this occurrence, and this type of reaction was not seen again.

Our study, designed to look at attractiveness and hatch rate, did not determine whether or not sufficient methoprene would be leeched from the oviposition substrate

into the larval habitat (water) to reduce the percent of adult emergence. This is an experiment that should be done, because through casual observation, it appears that there may be such a residual effect.

CONCLUSION

Statistical analysis of all egg counts using ANOVA failed to demonstrate a significant difference in the number of eggs deposited at any particular site in the cage or at any particular concentration of methoprene or the control. The ANOVA probability generated was greater than the five percent needed to reject the null; therefore, the null hypothesis that methoprene is not an oviposition attractant was not rejected.

There was no significant difference in hatch rate shown among the various treatments or the control. The probability generated by ANOVA was much greater than the five percent needed to reject the null; therefore, the null hypothesis that methoprene has no affect on the hatch rate of *Aedes triseriatus* could not be rejected.

REFERENCES CITED

[1] Logan, T.M., Linthicum, K.J., Wagateh, J.N., Thande, P.C., Kamau, C.W. and Roberts, C.R. Pretreatment of Floodwater *Aedes* Habitats (Dambos) in Kenya with a Sustained-Release Formulation of Methoprene. *JAMCA* 6(4): 736-38. December, 1990.

[2] Floore, T.G., Rathburn, C.B., Jr., Boike, A.H., Jr., Rodriguez, H.M., and Coughlin, J.S. Small Plot Test of Sustained-Release Altosid® (Methoprene) Pellets Against *Aedes Taeniorhynchus* in Brackish Water. *JAMCA* 6(1): 133-34. March, 1990.

[3] Beehler, J.W. and Defoliart, G.R. A Field Evaluation of Two Suggested *Aedes triseriatus* Oviposition Attractants. *JAMCA* 6(4): 720-722. December, 1990.

[4] Grimstad, Paul R. and Walker, Edward D. *Aedes triseriatus* (Diptera; Culcidae) and LaCrosse Virus. IV. Nutritional Deprivation of Larvae affects the Adult Barriers to Infection and Transmission. *J. Med. Entomol.* 28(3): 378-386.

[5] Turell, M.J. and LeDuc, J.W. The Role of Mosquitoes in the Natural History of California Serogroup Viruses. California Serogroup Viruses. [Ed: Charles H. Calisher and Wayne H. Thompson]. New York: Alan R. Liss, Inc., 1983. Pgs. 43-55.

[6] Darsie, R.F., Jr. and Ward, R.A. Identification and Geographical Distribution of the Mosquitoes of North America, North of Mexico. Fresno, CA: American Mosquito Control Association, 1981.

[7] Thompson, W.H. Vector-Virus Relationships. California Serogroup Viruses. [Ed: Charles H. Calisher and Wayne H. Thompson]. New York: Alan R. Liss, Inc., 1983. Pgs. 57-66.

[8] Carpenter, Stanley J. and LaCasse, Walter J. Mosquitoes of North America. Berkeley and Los Angeles: University of California Press, 1955. Pgs. 255-257.

[9] Linley, John R. Scanning Electron Microscopy of the Egg of *Aedes (Protomacleaya) triseriatus* (Diptera: Culcidae). *J. Med. Entomol.* 26(5): 474-78, 1989.

[10] Arunagiri, C.K., Perera, L.P., Abeykoon, S.B., Peiris, J.S.M. A Serologic Study of California Serogroup Bunyaviruses in Sri Lanka. *Am. J. Trop. Med. Hyg.* 45(3): 377-382, 1991.

[11] Kappus, K.D. Monath, T.P., Kaminski, R.M. and Calisher, C.H. Reported Encephalitis Associated with California Serogroup Virus Infections in the U.S. 1963-1981. California Serogroup Viruses. [Ed: Charles H. Calisher and Wayne H. Thompson]. New York: Alan R. Liss, Inc., 1983. Pgs. 31-41.

[12] Hammon, W. and Reeves, W.C. California Encephalitis Virus. *California Medicine* 77(5): 303-309, November 1952.

[13] Reeves, W.C., Emmons, R.W. and Hardy, J.L. Historical Perspectives on California Encephalitis Virus in California. California Serogroup Viruses. [Ed: Charles H. Calisher and Wayne H. Thompson]. New York: Alan R. Liss, Inc., 1983. Pgs. 19-29.

[14] Yuill, T.M. The Role of Mammals in the Maintenance and Dissemination of LaCrosse Virus. California Serogroup Viruses. [Ed: Charles H. Calisher and Wayne H. Thompson]. New York: Alan R. Liss, Inc., 1983. Pgs. 77-87.

[15] Thompson, Wayne H., Kalfayan, B. and Anslow, R.O. Isolation of California Encephalitis Group Virus from a Fatal Human Illness. *Am. J. Epidemiol.* 81(3): 245-253, 1965.

[16] Francy, D.B. Mosquito Control for Prevention of California (LaCrosse) Encephalitis. California Serogroup Viruses. [Ed: Charles H. Calisher and Wayne H. Thompson]. New York: Alan R. Liss, Inc., 1983. Pgs. 365-375.

[17] Parry, James E. Control of *Aedes triseriatus* in LaCrosse, Wisconsin. California Serogroup Viruses. [Ed: Charles H. Calisher and Wayne H. Thompson]. New York: Alan R. Liss, Inc., 1983. Pgs. 355-363.

[18] Beehler, J.W. and Mulla, M.S. Effect of the Insect Growth Regulator Methoprene on the Ovipositional Behavior of *Aedes aegypti* and *Culex quinquefasciatus*. *J. Am. Mosquito Control Assoc.* 9(1): 13-16.

TABLE 1
NUMBER OF EGGS LAID AT OVIPOSITION SITES AND EGG HATCH RATE - *Aedes triseriatus*

	Control	.1	1	10
12/4/92 Cage 1	143 eggs Desiccated	68 eggs Desiccated	68 eggs Desiccated	266 eggs Desiccated
12/4/92 Cage 2	325 eggs Desiccated	335 eggs Desiccated	375 eggs Desiccated	205 eggs Desiccated
1/17/93 Cage 1	532 eggs 77% hatch	407 eggs 84% hatch	267 eggs 74% hatch	821 eggs 67% hatch
1/17/93 Cage 2	563 eggs 83% hatch	59 eggs 25% hatch	890 eggs 76% hatch	911 eggs 68% hatch
1/29/93 Cage 1	269 eggs 73% hatch	201 eggs 72% hatch	400 eggs 68% hatch	468 eggs 72% hatch
1/29/93 Cage 2	366 eggs 68% hatch	0 eggs	97 eggs 72% hatch	202 eggs 70% hatch
2/26/93 Cage 2	803 eggs 73% hatch	720 eggs 63% hatch	577 eggs 69% hatch	816 eggs 69% hatch
3/5/93 Cage 1	966 eggs 22% hatch	1117 eggs 31% hatch	1113 eggs 47% hatch	1569 eggs 12% hatch
3/5/93 Cage 2	259 eggs 76% hatch	260 eggs 40% hatch	600 eggs 24% hatch	278 eggs 27% hatch
4/4/93 Cage 1	134 eggs 57% hatch	275 eggs 68% hatch	238 eggs 60% hatch	22 eggs 14% hatch
4/22/93 Cage 2	195 eggs 82% hatch	286 eggs 83% hatch	199 eggs 79% hatch	236 eggs 67% hatch
5/27/93 Cage 1	278 eggs 82% hatch	545 eggs 75% hatch	670 eggs 84% hatch	433 eggs 68% hatch
5/27/93 Cage 2	681 eggs 96% hatch	1110 eggs 72% hatch	809 eggs 85% hatch	544 eggs 96% hatch
6/4/93 Cage 1	622 eggs 92% hatch	1040 eggs 83% hatch	427 eggs 82% hatch	604 eggs 90% hatch
6/4/93 Cage 2	463 eggs 90% hatch	337 eggs 42% hatch	360 eggs 82% hatch	449 eggs 90% hatch
TOTALS	**6,599 eggs 74.7% hatch**	**6,760 eggs 61.5% hatch**	**7,090 eggs 69.4% hatch**	**7,824 eggs 62.3% hatch**

FIGURE 1. DISTRIBUTION OF *AEDES TRISERIATUS* IN THE UNITED STATES

FIGURE 2. DISTRIBUTION OF STATES WITH HIGHEST REPORTED LAC ENCEPHALITIS CASES

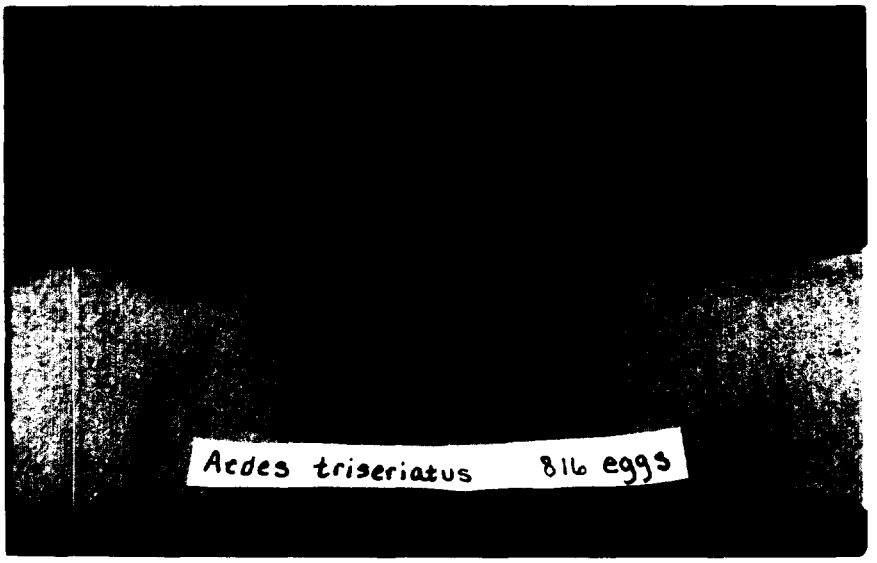


FIGURE 3. Paper toweling containing *Aedes triseriatus* eggs

Percent of Total Eggs Laid At Each Oviposition Site

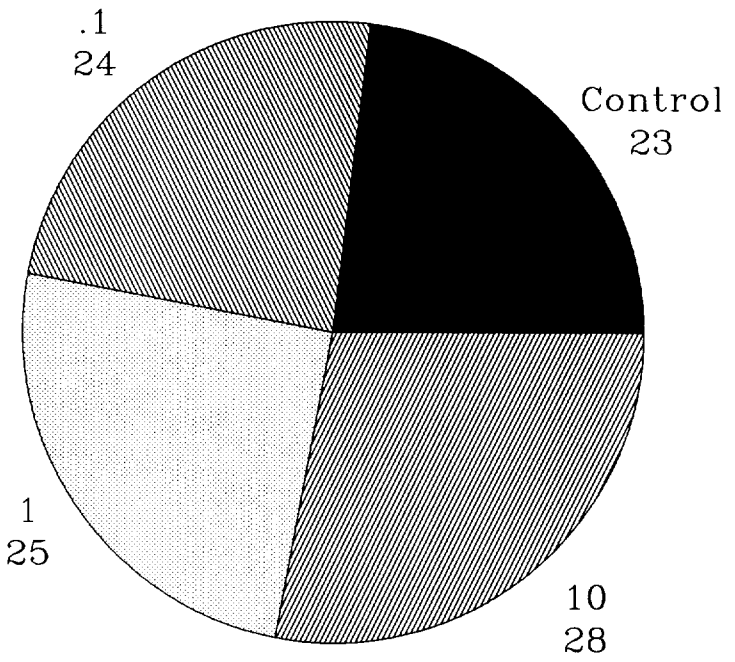
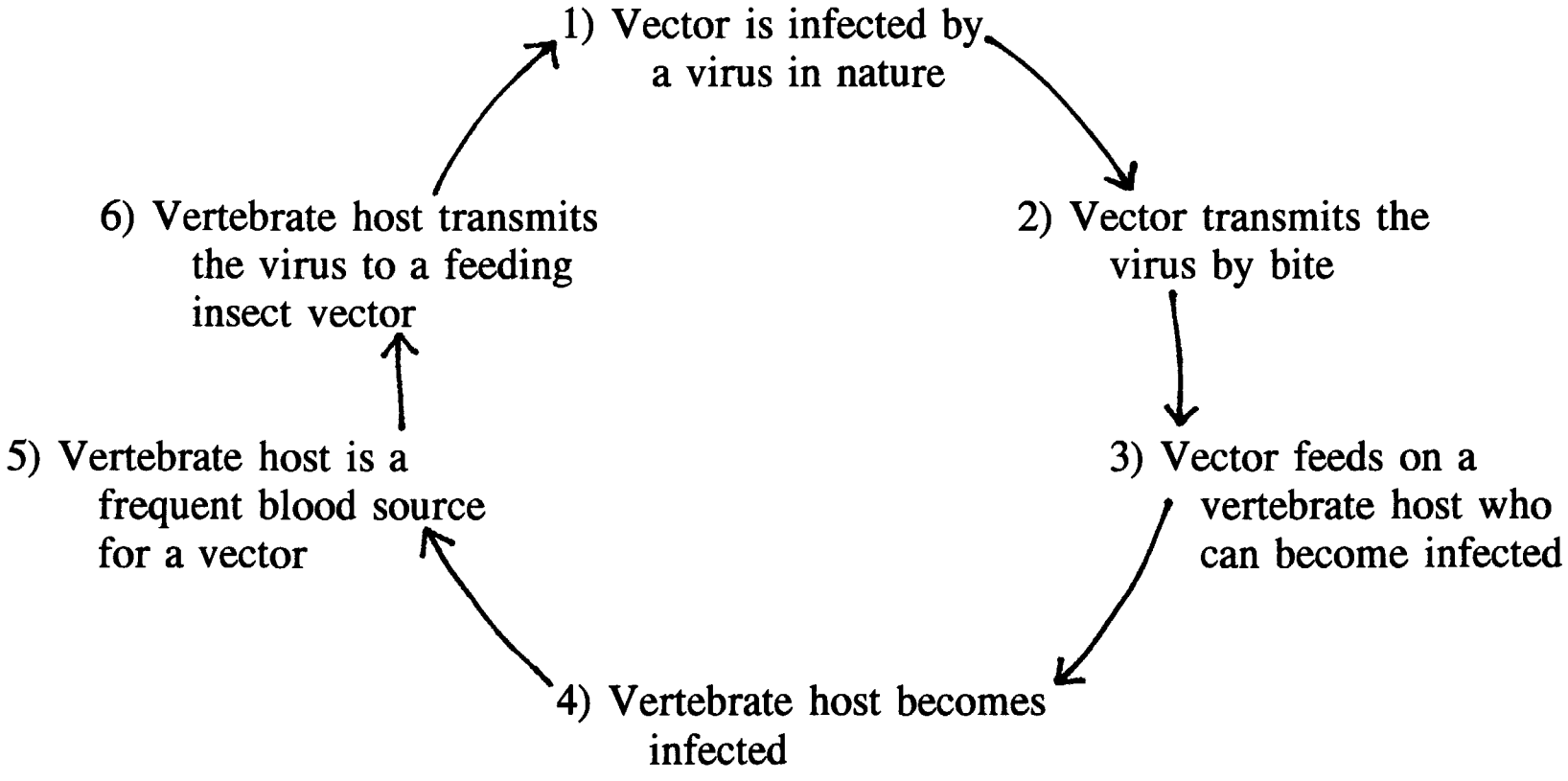


Figure 5

FIGURE 4. THE VECTOR-VIRUS CYCLE



Each Oviposition Site's Average Hatch Rate

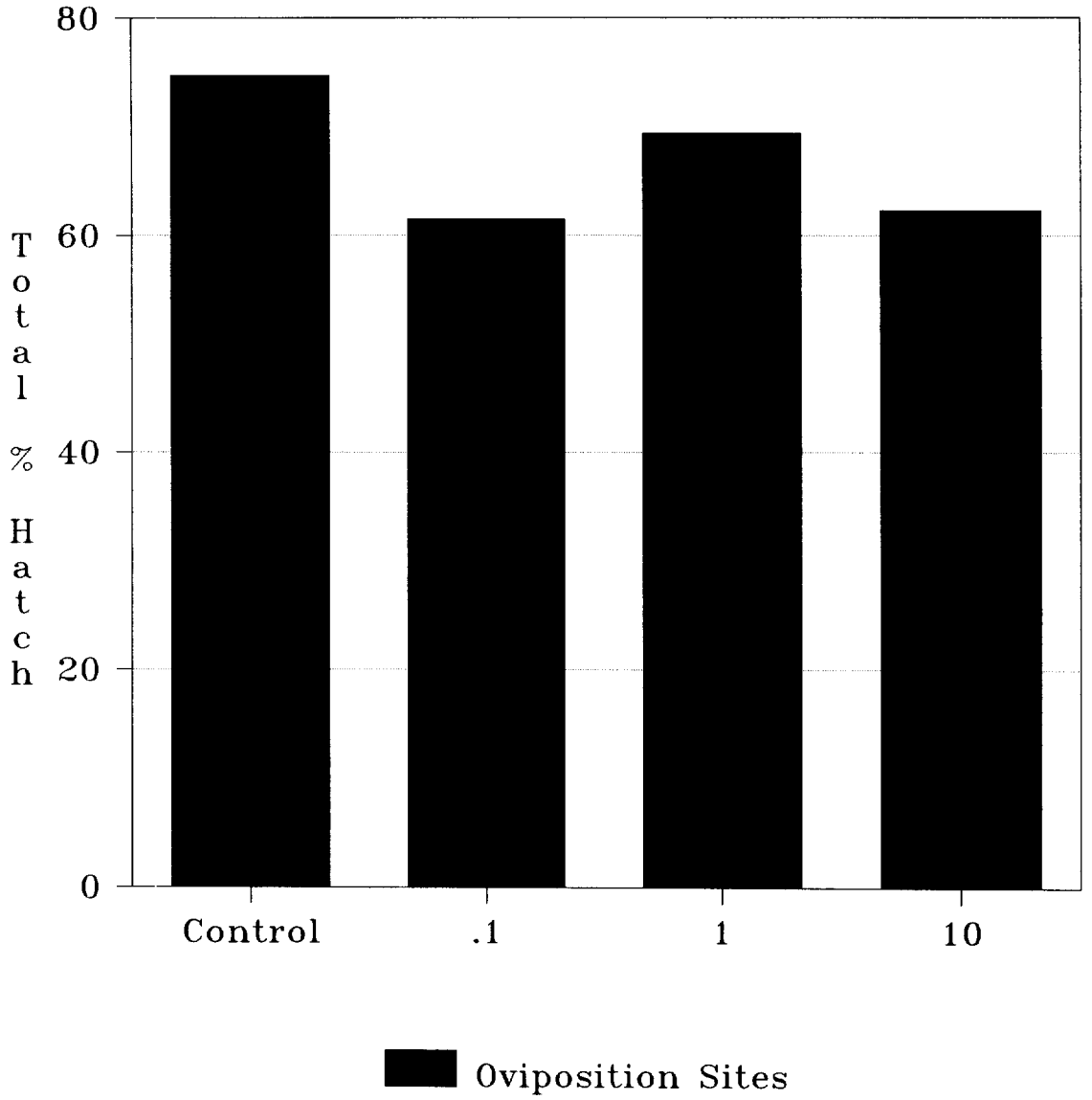


Figure 6

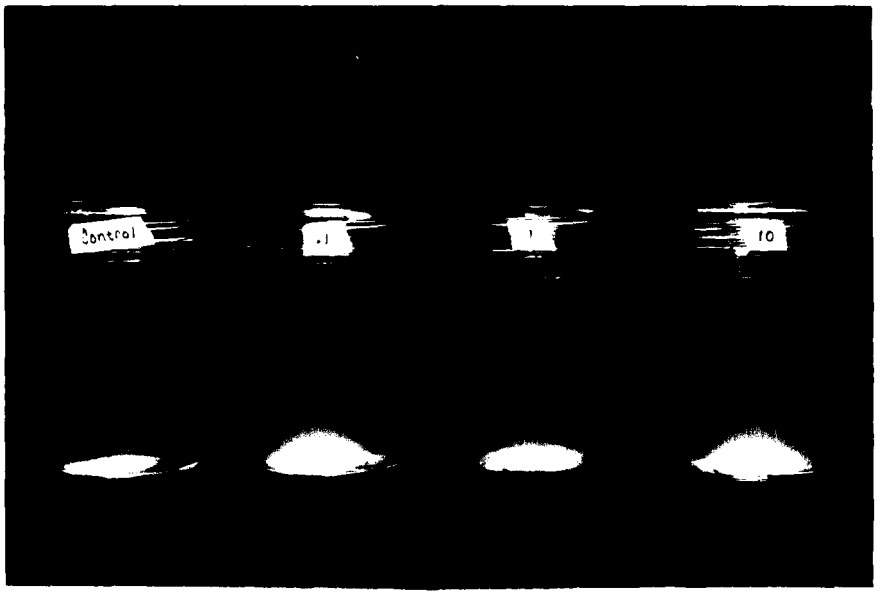


FIGURE 7. Water containing larvae from the four experimental oviposition sites: (left to right) control, .1x normal field application rate, 1x normal field application rate, and 10x normal field application rate. The water of the control batch is clear, while the water containing the three concentrations of methoprene is turbid.